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Maintaining multipotent trunk neural crest stem cells as self-renewing crestospheres

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ABSTRACT

Neural crest cells have broad migratory and differentiative ability that differs according to their axial level of origin. However, their transient nature has limited understanding of their stem cell and self-renewal properties. While an *in vitro* culture method has made it possible to maintain cranial neural crest cells as self-renewing multipotent crestospheres (Kerosuo et al., 2015), these same conditions failed to preserve trunk neural crest in a stem-like state. Here we optimize culture conditions for maintenance of avian trunk crestospheres, comprised of both neural crest stem and progenitor cells. Our trunk-derived crestospheres are multipotent and display self-renewal capacity over several weeks. Trunk crestospheres display elevated expression of neural crest cell markers as compared to those characteristic of ventrolateral neural tube or mesodermal fates. Moreover, trunk crestospheres express increased levels of trunk neural crest-enriched markers as compared to cranial crestospheres. Finally, we use lentiviral transduction as a tool to manipulate gene expression in trunk crestospheres. Taken together, this method enables long-term *in vitro* maintenance and manipulation of multipotent trunk neural crest cells in a premigratory stem or early progenitor state. Trunk crestospheres are a valuable resource for probing mechanisms underlying neural crest stemness and lineage decisions as well as accompanying diseases.

1. Introduction

The neural crest is a multipotent stem cell population that forms essential structures of the vertebrate body. Arising within the neuroectoderm after gastrulation, premigratory neural crest cells in the dorsal neural tube express numerous transcription factors including *FOXD3*, *TFAP2* and *SOXE* (Khudyakov and Bronner-Fraser, 2009). Around the time of neural tube closure, neural crest cells delaminate from the neural tube by undergoing an epithelial-to-mesenchymal transition (EMT), a feature shared with metastatic cancer cells, and then migrate extensively to populate distant sites in the embryo. In their final sites, neural crest cells differentiate into more than thirty different cell types, the range of which varies according to their anterior to posterior axial level of origin.

Elegant quail-chick grafting experiments (Ayer-Le Lievre and Le Douarin, 1982) have shown that different populations of neural crest cells arise at different levels of the body axis designated from anterior to posterior as cranial, vagal, trunk and sacral. These four subdivisions all share the ability to form cells of the peripheral nervous system, melanocytes and smooth muscle (Bittencourt et al., 2013; Bronner-Fraser and Fraser, 1988), but also give rise to axial level-specific features. For example, facial bone/cartilage arise from cranial neural crest (Ayer-Le Lievre and Le Douarin, 1982) whereas chromaffin cells of the adrenal medulla only arise from the trunk (Vega-Lopez et al., 2018).

The stem cell properties of neural crest cells make them a cell type of interest in regenerative medicine as well as a possible cell of origin for neural crest-derived tumors and birth defects (Vega-Lopez et al., 2018), highlighting the importance of understanding the regulatory

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mechanisms underlying neural crest stemness. To address this, we recently reported establishment of crestospheres for cranial neural crest cells. These are self-renewing multipotent primary neural crest cultures derived from either chick embryos or human embryonic stem cells (Kerosuo et al., 2015). Crestospheres maintain neural crest cells as premigratory neuroepithelial cells and differ from previous culture techniques which induce spontaneous differentiation (Baroffio et al., 1988; Curchoe et al., 2010; Lee et al., 2007; Stemple and Anderson, 1992; Trentin et al., 2004). Under crestosphere conditions, premigratory neural crest cells retain multipotency on a clonal level (Kerosuo et al., 2015). Given that there are known axial level differences between neural crest populations and different signaling cascades that determine the anterior to posterior patterning of the vertebrate body, it is not surprising that the established cranial crestosphere conditions do not support maintenance of trunk-derived neural crest cells.

To circumvent this problem, here, we provide a modified protocol that enables long term growth of trunk crestospheres as epithelial spheres under stem cell-promoting culture conditions. We show that trunk crestospheres in culture are multipotent with the ability to differentiate into a wide variety of neural crest-derived lineages including neuronal, smooth muscle, glia/astrocyte and cartilage. In addition, these crestospheres possess self-renewal capacity over several weeks. Trunk-derived crestospheres express general markers of the premigratory neural crest as well as genes specific to the trunk level, as shown by quantitative PCR, *in situ* hybridization and immunohistochemistry. Importantly, as a useful tool for gene expression manipulation, trunk neural crest-derived crestospheres can be efficiently transduced with fluorescently labelled lentiviral vectors. Thus, they hold great promise as an *in vitro* model for examining trunk neural crest-associated birth defects and malignancies such as the childhood tumor form neuroblastoma and adult tumor forms paraganglioma and pheochromocytoma.

2. Material and methods

2.1. Chick embryos

Chick embryos were obtained from commercially purchased fertilized eggs and incubated at 37.5 °C/100°F until they reached desired developmental stages. For trunk cultures, we used stage 13-/14 + (17–21 somite stage) embryos. For the cranial cultures used as controls, stage 8-/9 (4–7 somite stage) embryos were used, staged according to the criteria of Hamburger Hamilton (HH) (Hamburger and Hamilton, 1951). In a few cases, we tested cranial cultures from later stages when most of the neural crest has already migrated from the neural tube (*i.e.* HH10 +).

2.2. Neural tube dissection

Embryos at designated somite stages were collected from the eggs by using Whatman filter paper. Embryos were placed in the center and transferred to Ringer's balanced salt solution (Solution-1: 144 g NaCl, 4.5 g $CaCl{\cdot}2H_2O$, 7.4 g KCl, ddH_2O to 500 ml; Solution-2: 4.35 g $Na_2HPO_4{\cdot}7H_2O$, 0.4 g KH_2PO_4, ddH_2O to 500 ml (adjust final pH to 7.4)). Embryos were detached from the filter paper and placed ventral side up to cut out the endoderm by “cutting from the ventral midline”. Neural tubes from respective axial levels were carefully dissected out, and all neighboring mesoderm and notochord tissue was removed. Isolated neural tubes were transferred to a small volume (50 µl) of sterile PBS on ice. For cranial-derived cultures used as controls, the very anterior tip was excluded, and the neural tube was dissected until the first somite level as previously described (Kerosuo et al., 2015). For trunk-derived cultures, the neural tube was dissected between somites 10–15, and neighboring somites were removed to highest degree possible. Pools of neural tubes from 4 to 6 embryos were used for each culture.

Table 1
Culture medium optimized for trunk crestospheres.

Medium (NC)	C_{Final}
DMEM (with P/S)	
Chicken embryo extract	7.5%
B27	1 ×
IGF-I	20 ng/ml
FGF	20 ng/ml
Retinoic Acid	180 nM
BMP-4	25 ng/ml
DMEM	Corning #10-013-CVR, with 4.5 g/L Glucose
Chick Embryo Extract (CEE)	MP Biomedicals #092850145
B27	Life Technologies #12587-010 – 50 ×
Human recombinant IGF-I	Sigma Aldrich #SRP3069 – in ddH2O + 5% Trehalose in PBS (1:1)
Human recombinant bFGF	Peprotech – in Tris 5 mM pH7.6
Retinoic acid	Sigma Aldrich #R2625-10 mM in DMSO
BMP-4	Peprotech #120-05 – in citric acid (pH3.0): BS with 2 mg/ml BSA (1:1)

2.3. Cell culture

Pooled neural tubes were mechanically dissociated (~ 30 times until clumps of ~ 50–100 cells were formed) and transferred to NC medium (DMEM with 4.5 g/L glucose, 7.5% chick embryo extract, 1X B27, basic fibroblast growth factor (bFGF, 20 ng/ml), insulin growth factor -I (IGF-I, 20 ng/ml), retinoic acid (RA; 60 nM for cranial and 180 nM for trunk, respectively), and with or without 25 ng/ml BMP-4 in low-adherence T25 tissue culture flasks (2 ml (cranial) or 1.5 ml (trunk) per flask in upright position)). See Table 1 for complete culture conditions. During the course of 7–14 days, the total volume of media was incrementally increased to a maximum of V_{tot} = 10 ml. Due to rapid degradation, retinoic acid and BMP-4 were re-added to the culture medium volume every 2–3 days, and the spheres were also gently dispersed by pipetting up and down ~ 10–20 times against the culture flask wall every 2–3 days.

2.4. RNA extraction and quantitative real-time PCR

Total RNA was extracted using the RNAqueous Micro Kit (Ambion) and eluted in 20µl elution solution. cDNA synthesis using random primers and qRT-PCR was performed as previously described (Mohlin et al., 2015). Relative mRNA levels were normalized to expression of two reference genes (*18S, 28S*) using the comparative Ct method (Vandesompele et al., 2002). See Supplementary Table 1 for primer sequences.

2.5. Whole mount in situ hybridization of embryos and crestospheres

For whole mount *in situ* hybridization, embryos were fixed overnight in 4% paraformaldehyde (PFA) at + 4 °C, washed in PBS containing 0.1% Tween 20 with 0.1% DEPC (DEPC-PBT), dehydrated in a methanol (MeOH)/PBT series at room temperature and kept in 100% MeOH at −20 °C until use. *In situ* hybridization was performed as previously described (Acloque et al., 2008); briefly, embryos were rehydrated back to 100% PBT and prehybridized in hybridization buffer for 2 h at 70 °C. Embryos were then hybridized with Digoxigenin (DIG)-labelled probes overnight at 70 °C. The next day, embryos were washed in prehybridization buffer multiple times before switching to Maleic Acid Buffer (MAB) with 0.1% Tween 20 (MABT). Embryos were then blocked in MABT solution with 10% Boehringer Blocking Reagent and 10% Sheep Serum for 2 h and incubated with an anti-DIG antibody (1:2000) (Roche) in blocking solution overnight at 4 °C. On day 3, embryos were washed in MABT throughout the day and then switched into Alkaline phosphatase buffer (NTMT; 100 mM NaCl, 100 mM Tris-Cl (pH 9.5), 50 mM MgCl2, 1%Tween-20) before visualizing the signal using nitroblue tetrazolium (NBT) and

5-Bromo-4-chloro-3-indolyl phosphate (BCIP) solutions (Roche). Embryos were fixed in 4% PFA for 20 min when they reached the desired state and dehydrated in MeOH to be stored at −20 °C. Images of whole mount embryos were taken using an Axioskop2 (Zeiss) microscope equipped with Axiovision software, and a region between somites 10–15 was dissected and embedded in blocks of gelatin for transverse sectioning at 20 μm using a cryostat. Detailed information of antibodies can be found in Supplementary Table 2.

Similarly, crestospheres were fixed in 4% PFA for 30 min at RT or overnight at + 4 °C and washed in DEPC-PBT. Samples were then gradually dehydrated by bringing them to 100% MeOH and kept at −20 °C for a minimum of 2 h. *In situ* hybridization was performed as described above (Acloque et al., 2008). Avian DIG-labelled hybridization probes for *SOX10, SOX9, PAX7, SNAI2, FOXD3* and *SOX2* and were cloned by using chicken cDNA as previously described (Khudyakov and Bronner-Fraser, 2009).

2.6. Cryosections and immunohistochemistry

Crestospheres were fixed in 4% paraformaldehyde for 15 min at RT and washed three times in TBS-T (TBS + Ca^{2+} supplemented with 0.1% Triton-X). For embedding, spheres were incubated in a sucrose gradient (5% sucrose for 10 min and 15% sucrose for 10 min, all at RT) followed by incubation in 7.5% gelatin over night at 37 °C, then placed into a mold and snap frozen. Embedded spheres were cryosectioned at 12 μm.

For immunofluorescence, whole spheres were blocked in 10% goat serum in TBS-T or 10% donkey serum in PBS-T for 4 h at RT and washed twice in TBS-T for 30 min before incubation with primary antibody (mouse anti-PAX7, Hybridoma Bank) diluted in block solution over night at + 4 °C. After two times washing for 30 min in TBS-T, crestospheres were incubated with secondary antibody (anti-mouse Alexa Fluor) and DAPI (diluted in blocking solution) for 4 h at RT. SOX9 immunostaining was performed on sections following the same protocol followed by anti-rabbit Alexa Fluor for 1 h at RT as secondary antibody. Slides were washed in TBS-T and mounted. Detailed information of antibodies can be found in Supplementary Table 2.

2.7. Self-renewal / primary sphere assay

Crestospheres were dissociated into single cells using Accutase (Sigma Aldrich; incubation at 37 °C for 40 min with one minute of pipetting every 10 min), and one (1) or five (5) cell(s) were manually picked using a p10 pipette tip under the microscope. Single cells were transferred to 96-well plates prepared with 100 μl of NC medium supplemented with RA and BMP-4. New NC medium supplemented with RA and BMP-4 (V_{total} = 100 μl) was added every 2–3 days to avoid wells to dry out. The absolute number of spheres formed in each well was quantified manually under the microscope, where clusters of more than ten cells were counted as spheres. Cultures initiated with one cell/well were counted after one week followed by another counting after the second week without dissociation of the spheres during the culture period. The second week thus reflected the number of secondary spheres formed from the primary spheres. Spheres seeded in a dilute concentration of five cells per well were counted after one week of culture. Results obtained for 1 cell/well conditions (T = 1 week and T = 2 weeks) were derived from three individual crestosphere cultures (n = 3) and five wells were analyzed per culture (n = 15 wells in total). Similarly, results obtained for 5 cells/well conditions were derived from two individual crestosphere cultures (n = 2) and n = 5 wells were analyzed per culture (n = 10 wells in total).

2.8. Differentiation assay

Crestospheres were mechanically dissociated into smaller clusters and seeded on coated cover slips (0.1 μg/μl poly-D-lysine, BD Biosciences). Crestospheres were incubated for 7 days in DMEM + 1% fetal calf serum (FCS) (α-SMA; GFAP; RUNX2) or DMEM + 1% N2 + 1% FCS+ 1% CEE (BLBP; TUJ1). Medium was changed once during the incubation time. Cells were fixed in 4% PFA for 15 min at RT and cover slips blocked in 5% Goat serum + 0.3% Triton-X in PBS-DEPC (α-SMA; TUJ1; RUNX2) or 0.3% Triton-X in PBS/3% BSA (GFAP; BLBP) for 1 h at RT. Before incubation with BLBP antibody, antigen retrieval was performed by brief boiling in 10 mM trisodium citrate pH6. Incubation with primary antibodies was performed at + 4 °C over night. After washing in PBS, incubation with secondary antibodies diluted in corresponding blocking solution was performed in combination with DAPI (Dako) for 1 h at RT. Detailed information of antibodies can be found in Supplementary Table 2.

2.9. Lentiviral transduction

Crestospheres were dissociated into single cells / small clusters using Accutase (Sigma Aldrich; incubation at 37 °C for 20–40 min with one minute of pipetting every 10 min) and seeded at high density in 150 μl culture medium in 24-well plates. Cells were directly transduced with increasing concentrations of pCIG3-GFP (pCMV-IRES-GFP version 3, kind gift from Felicia Goodrum (Addgene plasmid # 78264) (Caviness et al., 2014)). Medium was changed twice a day for four consecutive days. GFP expression was analyzed 96 h post-transduction using an Olympus inverted fluorescence microscope. Virus titers were optimized and calculated using sphere growing neuroblastoma patient-derived xenograft cells (Persson et al., 2017) due to the difficulty in obtaining pure single cell suspensions of trunk crestosphere cultures. Two titers were tested; 2.1*10^8 TU/ml and 2.3*10^8 TU/ml; TU, transducing units.

2.10. Resources

Essential resources and reagents are listed in Key Resource Table.

3. Results & discussion

3.1. Optimization of the correct trunk premigratory axial level

Cranial crestospheres grow as three-dimensional spheres under conditions optimized for neural tubes from chick embryos as well as human embryonic stem (ES) cells; these spheres retain their self-renewal capacity and multipotency for up to seven weeks in culture (Kerosuo et al., 2015). Here, we sought to devise modifications that would enable growth and maintenance of premigratory neural crest cells from more posterior axial levels to provide a tool for studies on trunk neural crest stemness as well as trunk-derived neurocristopathies.

To ensure that the proper stage from which to derive crestosphere cultures was used, we performed *in situ* hybridization on whole mount embryos using neural crest markers *SOX10*, *FOXD3*, *PAX7*, *SOX9*, *SNAI2* and the neuroepithelial marker *SOX2* (Fig. 1A-F). As expected, the endogenous expression patterns showed that the trunk neural crest was premigratory at the level adjacent to somites 10–16 in stage HH13-/14 embryos.

We tested our cranial crestosphere protocol on dissociated cell clusters of trunk neural tubes derived from chick embryos at several stages (HH10 +, HH12, HH13/14), and confirmed that 17–21 somite stage (HH13/14) embryos was the optimal developmental stage for trunk crestosphere culture establishment (Fig. 2A). With HH13/14 embryos, spheres formed rapidly after tissue dissociation and expanded accordingly during *in vitro* culture, whereas trunk crestospheres derived from HH10 + or HH12 embryos grew slower and expressed lower levels of neural crest markers (data not shown).

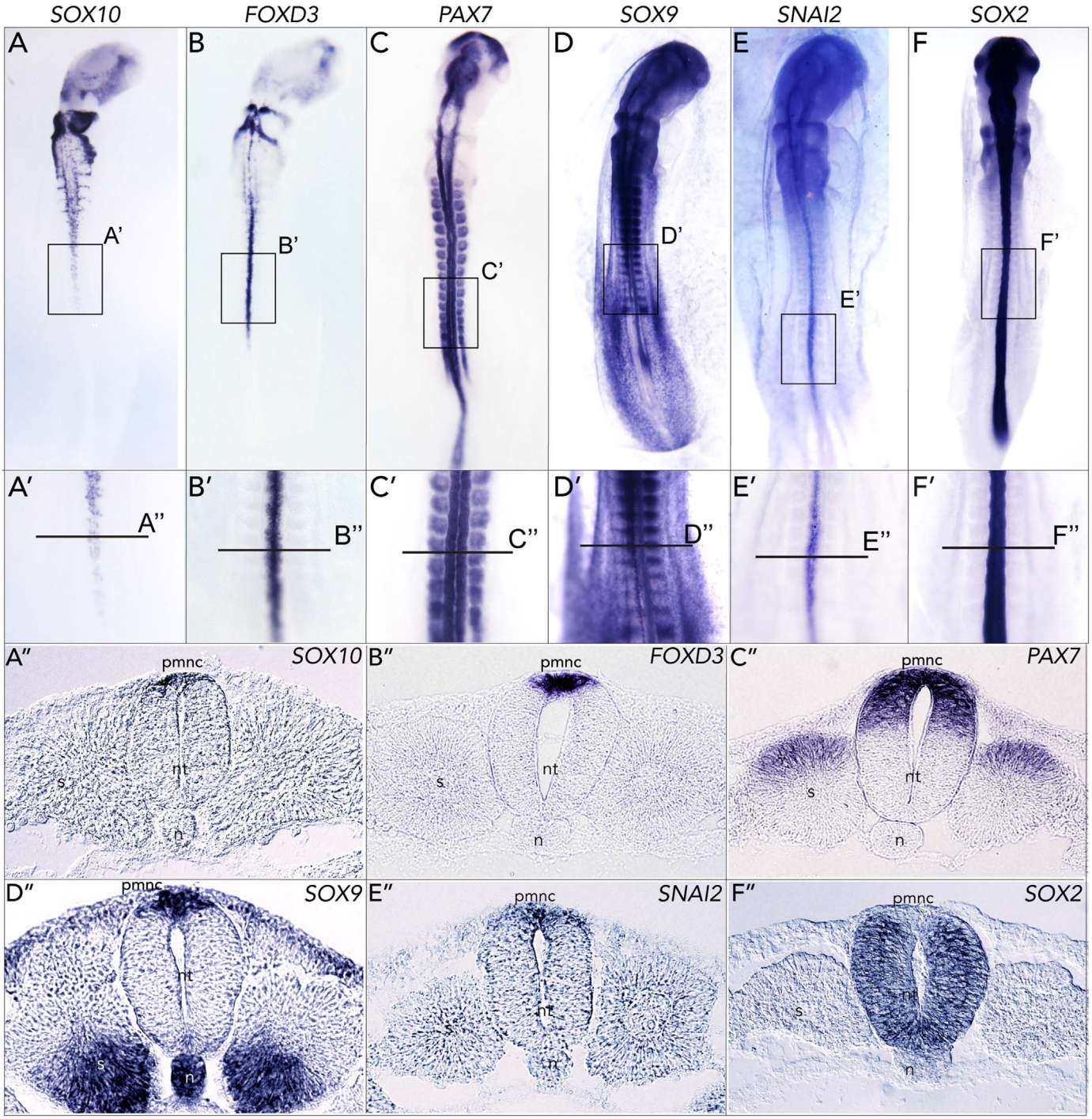

Fig. 1. Endogenous expression of premigratory neural crest markers in the posterior axial levels. **(A-F)** Whole mount *in situ* hybridization of HH13–14 embryos. (A'-F') Inserts showing the neural tube at the respective somite level that was dissected for trunk crestosphere cultures. (A''-F'') Transverse sections of the neural tubes at the level that was used for trunk crestosphere cultures. nt, neural tube; n, notochord; s, somite; pmnc, premigratory neural crest.

3.2. Trunk neural crest cells do not survive in conditions optimized for cranial crestospheres

Although it was initially possible to establish trunk crestospheres under culture conditions optimized for cranial crestospheres (Kerosuo et al., 2015), trunk crestospheres failed to thrive and expand long term. All cultures resulted in less than 25 spheres each during the entire three-week culture period (n = 4). In comparison, all three simultaneously established cranial cultures produced 100 + spheres (n = 3).

Given the known importance of retinoic acid (RA) in posteriorizing the body axis as well as in caudal neural crest development (Kudoh et al., 2002; Martinez-Morales et al., 2011; Retnoaji et al., 2014), we increased the amount of RA in the culture medium from 60 nM (Kerosuo et al., 2015) to 180 nM. Visual observation of sphere growth suggested that 180 nM of RA was optimal for successful expansion of trunk-derived crestospheres. All cultures resulted in more than 50–100 spheres (n = 6). Despite the additional supply of RA, trunk-derived crestosphere cultures continued to grow more slowly than cranial-derived cultures. We therefore optimized the starting culture volume by further decreasing it by 25% from 2 ml used for cranial crestospheres (Kerosuo et al., 2015) (n = 7) to 1.5 ml NC medium (n = 8). This decrease in starting culture volume had a

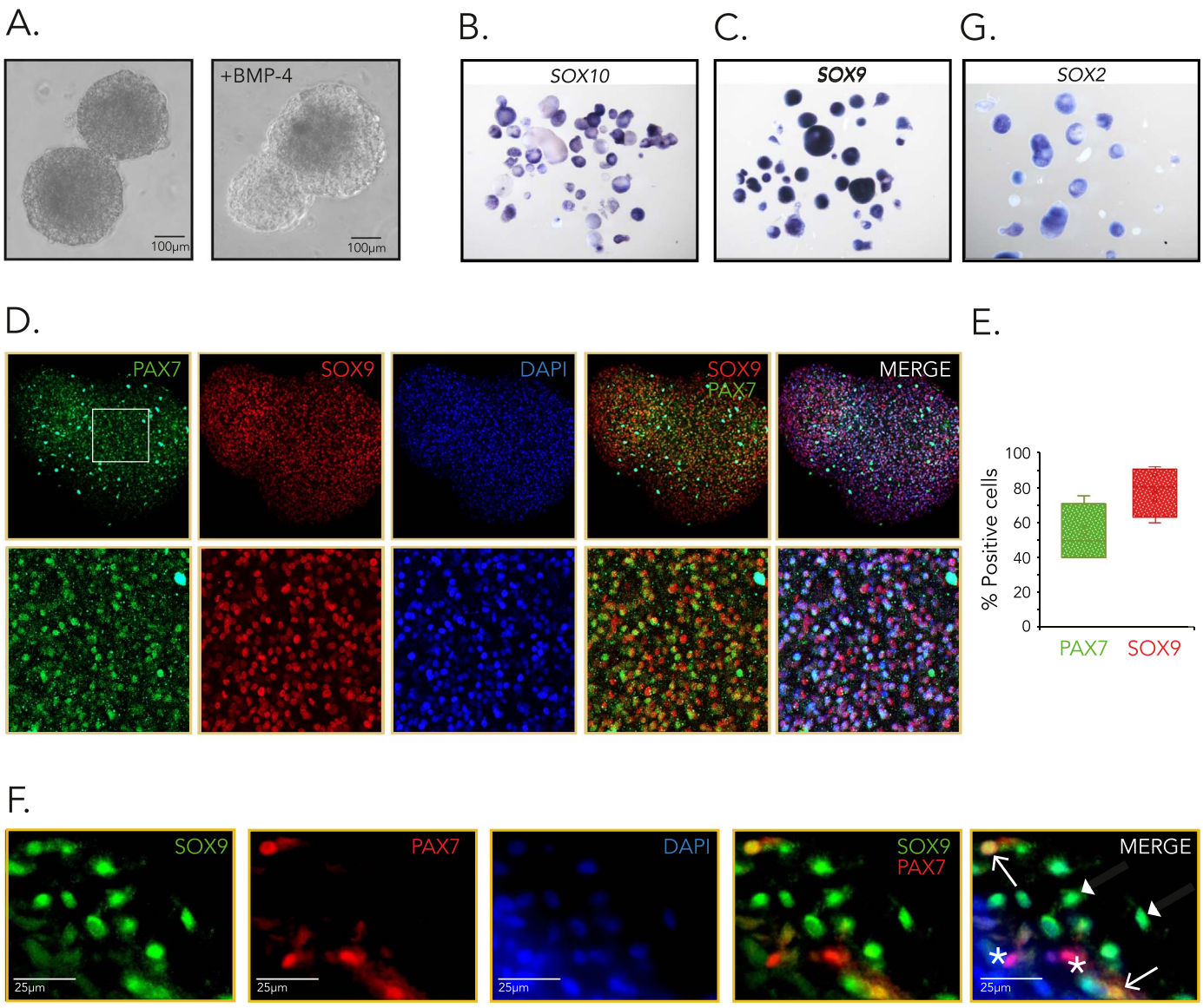

Fig. 2. Trunk crestospheres are enriched for neural crest marker expression. **(A)** Trunk crestospheres cultured without or with BMP-4 *in vitro* grow in a similar fashion. **(B)** *In situ* hybridization for *SOX10* on trunk crestospheres. **(C)** *In situ* hybridization for *SOX9* on trunk crestospheres. **(D)** PAX7 (green) and SOX9 (red) double immunostaining of a trunk crestosphere shows that the majority of cells express both markers. Please note that the large bright green spots are not specific staining. **(E)** Quantification of PAX7 and SOX9 positive cells, respectively. Four individual spheres (n = 4) were counted containing 1415 cells in total. **(F)** High magnification from a double PAX7 (red) and SOX9 (green) immunostained cryosection. Arrows indicate double positive cells, arrowheads indicate SOX9 single positive cells and asterisks indicate PAX7 single positive cells. **(G)** *In situ* hybridization for *SOX2* on trunk crestospheres.

positive impact on trunk crestosphere expansion, suggesting that the crestospheres condition their own medium by producing additional essential growth factors. The number of spheres in each culture reached ~50 in less than 48 h (n = 3), equivalent to that observed for cranial-derived spheres under optimal cranial crestosphere conditions. In similarity to previously established cranial crestospheres (Kerosuo et al., 2015), trunk crestosphere cultures grew and expanded *in vitro* for 6–7 weeks, after which they were still viable but virtually stopped to proliferate.

3.3. Trunk crestospheres are enriched for neural crest cells

Next, we performed *in situ* hybridization to test expression of neural crest markers in our established sphere cultures. Trunk crestospheres displayed strong expression of the early neural crest specifier genes *SOX10* and *SOX9* (Fig. 2B and C, respectively), that continue to be expressed in the migratory cells after EMT, which confirmed enrichment of neural crest cells. We applied immunohistochemistry to examine expression of PAX7, another early neural crest marker that starts to be expressed already during induction at the neural plate border, and SOX9. Furthermore, even though both PAX7 and SOX9 are expressed outside the neural crest domain in the neighboring tissues (Fig. 1C' and D', respectively) their expression pattern is overlapping only in the neural crest. Therefore, we performed dual immunostaining to further confirm the neural crest identity of trunk crestospheres. As expected (Kerosuo et al., 2015; Lignell et al., 2017), the vast majority of cells expressed both markers even though the intensity of the expression varied from cell to cell (Fig. 2D). Quantification of PAX7 + and SOX9 + cells showed that in average 54% (PAX7) and 78% (SOX9) of crestospheres were positive for these markers (Fig. 2E). Importantly, the Pax7 antibody in general gave a weaker staining than the Sox9, which may slightly impact the quantification. We also used high magnification imaging on cryosections, which confirmed the heterogenous expression pattern with some cells expressing only one or none of the markers, whereas others expressed both (Fig. 2F). Finally, we used *in situ* hybridization to detect expression of SOX2 (Fig. 2G). In

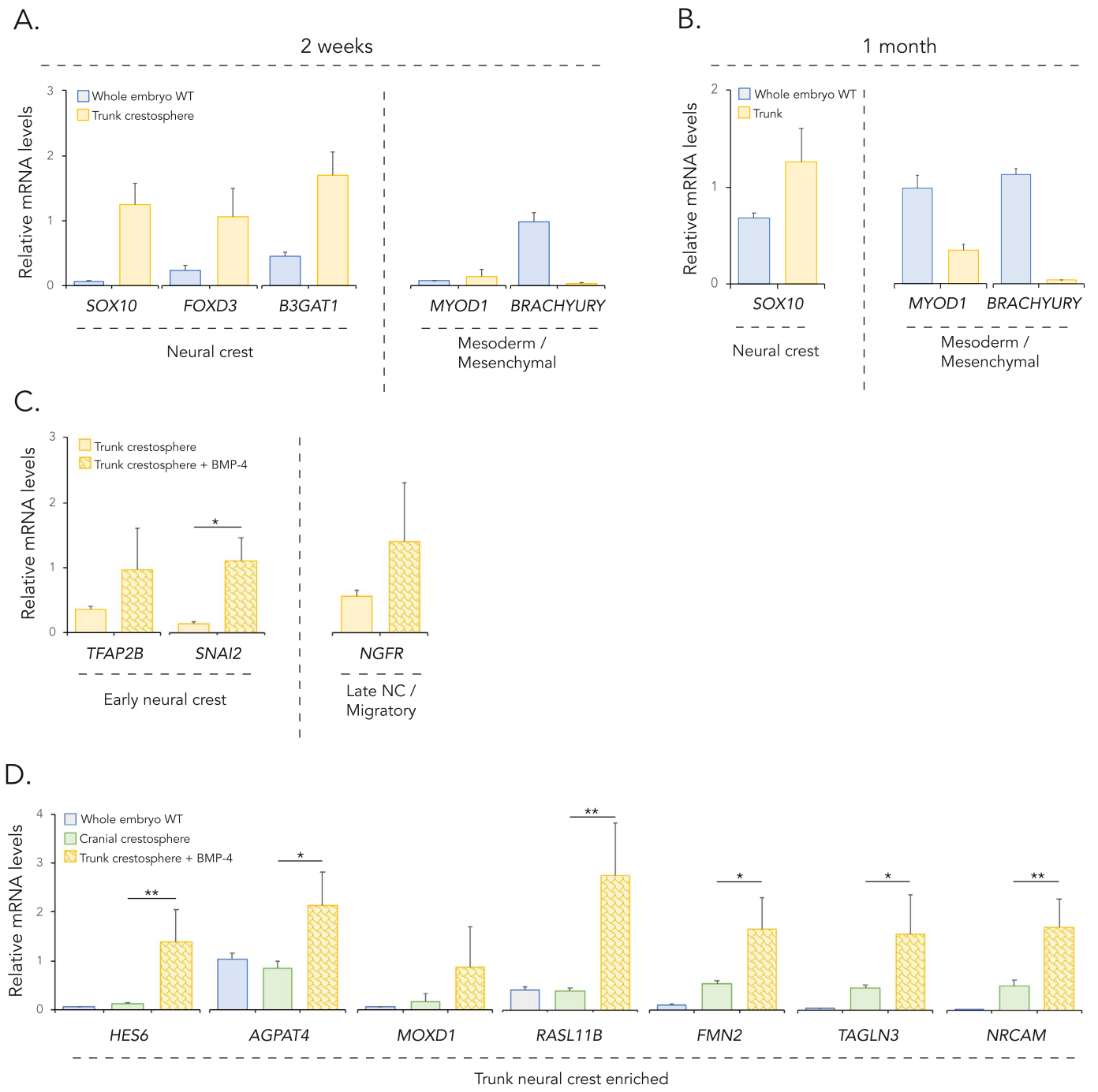

Fig. 3. Quantitative RT-PCR shows enriched neural crest gene expression in trunk crestospheres. **(A)** Relative mRNA expression of neural crest- and mesodermal- associated genes comparing trunk crestosphere cultures (n = 3) to stage HH10 whole embryo wild type (WT) (n = 1, pool of three embryos) at 2 weeks of culture. **(B)** Relative mRNA levels of neural crest and mesodermal markers in trunk crestospheres at 3.5 weeks of culture (n = 1) compared to stage HH13 whole embryo wild type (WT) (n = 1, pool of three embryos). **(C)** Relative mRNA expression of neural crest-associated genes comparing trunk crestospheres cultured with (n = 3) or without (n = 3) BMP-4. **(D)** Relative mRNA expression levels of trunk neural crest-enriched genes comparing trunk crestospheres cultured with BMP-4 (n = 3) to stage HH10 whole embryo wild type (WT) (n = 1, pool of three embryos) and cranial crestosphere cultures (n = 4). **(A-D)** Data is presented as mean of n = 3–4 ± SEM (from biologically independent repeats) or n = 1 ± SD (from technical triplicate), and significance between trunk crestospheres cultured with or without BMP-4 (**C**) and statistical significance for trunk and cranial crestosphere cultures (**D**) was calculated using two-sided student's *t*-test; *p < 0.05, **p < 0.01.

concordance with neural crest cells expressing low levels of SOX2 (Kerosuo et al., 2018; Roellig et al., 2017), we as well detected weak to moderate expression of *SOX2* further confirming the neuroepithelial nature of these spheres (Fig. 2G). Taken together, based on these data we conclude that our trunk crestosphere cultures are highly enriched for neural crest cells. However, we cannot rule out the possible existence of some "future CNS" neuroepithelial stem cells similar to what is observed in the developing neural tube.

3.4. Trunk-derived crestospheres consist of a combination of neural crest stem and progenitor cells

To further characterize gene expression patterns of the trunk crestosphere cultures, and to ensure lack of contamination from neighboring mesodermal cells, we used quantitative RT-PCR (qPCR) to address expression levels of neural crest markers relative to markers that reflect the presence of mesodermal fates. Consistent with *in situ*

hybridization results, trunk crestospheres cultured for 2 weeks (Fig. 3A) or one month (Fig. 3B) display increased expression of neural crest markers *SOX10* (Fig. 3A-B) and the premigratory marker *FOXD3* that becomes downregulated directly after EMT ((Lignell et al., 2017), Fig. 3A). We also detected increased expression levels of the "migratory only" neural crest marker *B3GAT1* (Fig. 3A; encoding the enzyme required for formation of the HNK1-epitope; expressed in avian neural crest), as compared to expression levels in whole wild-type (WT) embryos. These results show that trunk crestospheres consist of a mixture of cells at multiple neural crest developmental stages instead of purely premigratory neural crest cells, as shown in many spheroid cultures of different stem cell types (Jensen and Parmar, 2006; Piscitelli et al., 2015). Meanwhile, the relative expression levels of mesodermal genes *MYOD1* and *T* (T brachyury transcription factor) were low (Fig. 3A-B). These results confirm the primarily neural crest identity of trunk crestospheres and rule out contamination by mesoderm-derived cells.

3.5. Addition of BMP-4 to the culture medium promotes neural crest maintenance and expansion

During normal development BMP-4 is secreted by the dorsal neural tube at the premigratory stage to promote neural crest fate (Basch et al., 2006; Hackland et al., 2017; Liem et al., 1995; Steventon et al., 2009), and later by the dorsal aorta, providing cues for neural crest cell migration as well as sympathoadrenal and chromaffin cell fate (Unsicker et al., 2013). We therefore compared expression of neural crest genes in trunk crestosphere cultures without (*i.e.* '*normal*' NC medium) or with BMP-4 supplement, testing whether addition of BMP-4 could increase relative expression levels of neural crest genes. By visual inspection, the crestospheres grew similarly with or without BMP4 (Fig. 2A). However, expression of the early neural crest marker genes *TFAP2B* and *SNAI2* increased (Fig. 3C). We also detected increased levels of *NGFR*, a marker expressed later by migrating and maturing peripheral neurons (Heuer et al., 1990) (Fig. 3C), possibly also causing fate bias towards peripheral nervous system cell types. We conclude that BMP-4 further enriched neural crest fate in the cultures and should be routinely added to the optimized trunk crestosphere culture medium (Table 1). Altogether, we show that increased concentrations of RA are crucial for trunk crestosphere culture expansion (Table 1), and that BMP-4 enhances expression of at least some neural crest marker genes (Fig. 3C).

3.6. Trunk-derived crestospheres are enriched for trunk neural crest genes

Next, we performed qPCR to determine relative expression of recently characterized genes associated with trunk neural crest (Murko et al., 2018). We observed enriched expression levels of these genes in trunk crestospheres as compared to cranial crestospheres, reflecting the maintenance of posterior axial levels in the trunk crestosphere cultures (Fig. 3D).

3.7. Trunk crestosphere-derived cells are multipotent

To ensure that our established trunk crestospheres maintain their multipotent stem-like nature in culture, we plated the crestospheres in differentiation promoting conditions for one week. Microphotographs revealed substantial differences in cellular morphology (Fig. 4A), considerably aberrant from the growth pattern of crestospheres in NC medium (Fig. 2A), but also intercellularly within the differentiated cell population. To determine which cellular lineages trunk crestosphere-derived cells committed to, we used lineage specific antibodies and, as expected (Vega-Lopez et al., 2017), detected smooth muscle (α-SMA) (Fig. 4B), and peripheral neurons (TUJ1) (Fig. 4C) and glial cells/astrocytes (GFAP, BLBP) (Fig. 4D-E, respectively). Surprisingly, we also detected a few cells expressing the cartilage marker RUNX2, a feature of the cranial neural crest (Fig. 4F), perhaps reflecting a difference between *in vitro* and *in vivo* potential. These results demonstrate that trunk crestospheres maintained potential for several lineages under the defined stem cell cultures, and thus indicate maintenance of multipotency.

3.8. Single trunk-derived crestosphere cells self-renew in culture

Another important stem cell-associated trait is the ability to self-renew. For this, we performed primary sphere assays over the course of two weeks. Quantification of the number of formed spheres (cell clusters containing ≅ or > 10 cells) showed that trunk crestosphere-derived single cells were able to self-renew in culture in single cell wells (Fig. 4G) and when seeded sparsely in multi-cell wells (5 cells/well, Fig. 4H). We allowed cells seeded in single cell wells to continue to self-renew for an additional week (t = 2 weeks in total). As expected, the number of spheres increased over time as shown by higher sphere numbers counted after the second week (Fig. 4I-J). These results were confirmed by a strong correlation at a population level ($R = 0.81$; Fig. 4K).

3.9. Trunk crestospheres can be efficiently transduced using lentiviral vectors

Genetic manipulation of crestospheres is one potential application for analysis on downstream effects of genes of interest (Dupin et al., 2018; Kerosuo and Bronner, 2016). However, our previous attempts using siRNAs have demonstrated low transfection efficiency (Kerosuo and Bronner, 2016). Therefore, we sought to determine whether trunk-derived crestospheres could be efficiently transduced using viral vectors to create stable phenotypes. In a proof-of-principle experiment, we used a GFP-tagged lentiviral vector (pCMV-IRES-GFP version 3; pCIG3) and transduced trunk crestospheres with increasing concentrations of the virus. Trunk crestospheres did not display toxic side effects after viral transduction and survived well at all concentrations investigated. By analyzing GFP expression in individual spheres using live fluorescent microscopy, we observed no major differences in GFP intensity between the different viral concentrations tested (Fig. 4L). To assess transduction efficiency, we compared *EGFP* expression in regularly grown crestospheres (CTRL) and pCIG3-GFP transduced crestosphere cultures. There was an in average 200-fold induction in *EGFP* expression in transduced spheres (Fig. 4M). These results show that trunk crestospheres can be efficiently transduced to create stable genotypes, overcoming the transient and inefficient nature of siRNAs.

4. Conclusions

Crestospheres are an excellent tool to study different aspects of neural crest stemness and differentiation (Dupin et al., 2018; Kerosuo and Bronner, 2016; Kerosuo et al., 2015). Given the known axial level differences in the ability of cranial and trunk neural crest cells to give rise to different cell types (Ayer-Le Lievre and Le Douarin, 1982; Simoes-Costa and Bronner, 2016), here we optimized previous cranial crestosphere conditions (Kerosuo et al., 2015) for maintenance of trunk-derived neural crest cultures that arise from more posterior axial levels, as illustrated in Fig. 5. Our protocol for trunk crestosphere cultures results in enrichment of neural crest specific genes as shown by *in situ* hybridization, immunostaining and qPCR, with little expression of mesodermal markers.

Considering that we have not performed quantitative analysis at a single cell level, relative expression levels provide only a guideline for the enriched cell types. The benefit of evaluating sphere identity with neural crest markers like FOXD3, SOX10 and SNAI2 is that they are not expressed elsewhere in the neural tube or the surrounding mesoderm in the embryo. Therefore, their expression can be directly

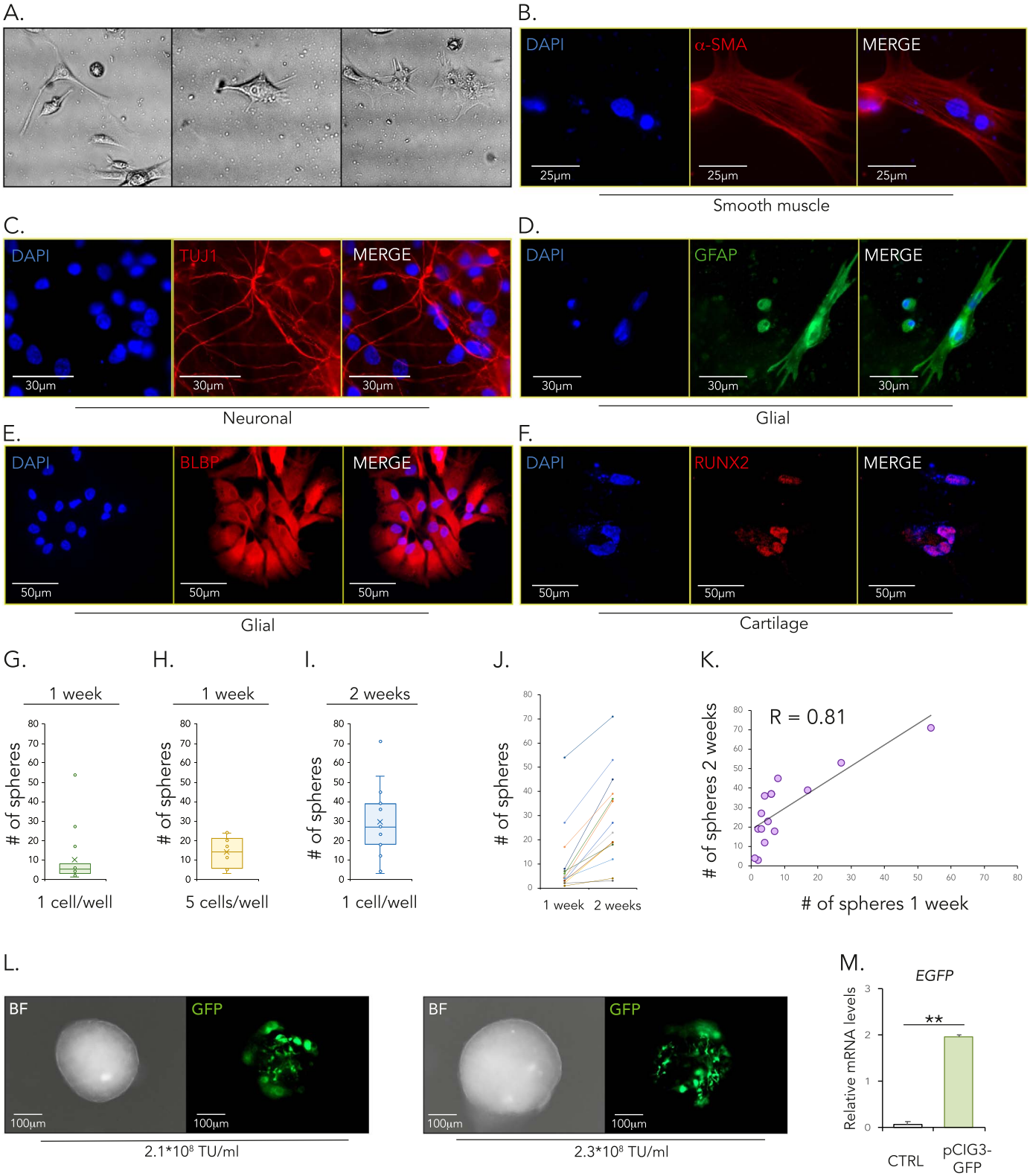

Fig. 4. Trunk crestospheres self-renew and are multipotent. (A) Microphotographs of trunk crestosphere-derived differentiated cells following adherent growth on poly-D-lysine coated plates and differentiation-inducing medium. **(B-F)** Immunofluorescent staining of markers representing smooth muscle (α-SMA, **B**), peripheral neuron (TUJ1, **C**), glial/astrocyte (GFAP [**D**], BLBP [**E**]) and cartilage (RUNX2, **F**) lineages following adherent growth on poly-D-lysine coated cover slips and differentiation-inducing medium. **(G)** Self-renewal capacity measured as number of spheres derived from single cells seeded as 1 cell/well. Number of spheres were quantified after one week of culture from 15 wells in total (five wells each from three individual crestosphere cultures). **(H)** Self-renewal capacity measured as number of spheres derived from single cells seeded at 5 cells/well. Number of spheres were quantified after one week of culture from 10 wells in total (five wells each from two individual trunk crestosphere cultures). **(I)** Self-renewal capacity measured as number of spheres derived from single cells seeded as 1 cell/well (G). Number of spheres was quantified after two weeks of culture; n = 15 wells from n = 3 trunk crestosphere cultures. **(J)** Primary sphere formation of each individual single cell seeded at 1 cell/well density assessed after one and two weeks of culture, displaying that each and every cell possesses self-renewal capacity. **(K)** Primary sphere formation of each individual single cell seeded at 1 cell/well density assessed after one and two weeks of culture, based on population level. Pearson correlation coefficient, R= 0.81. **(L)** GFP expression in individual trunk crestosphere cells transduced with different concentration of the virus shows similar transduction efficiency at all conditions. TU; Transducing Units. **(M)** Relative mRNA levels of *EGFP* comparing pCIG3-GFP transduced trunk crestosphere cultures and their non-transduced counterparts (CTRL). Data is presented as mean of n = 2 ± SEM, and significance was calculated using two-sided student's *t*-test; * *p < 0.01.

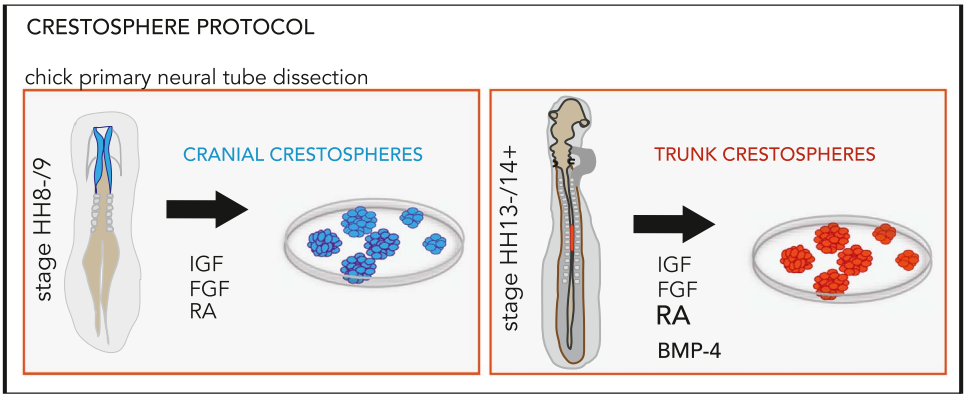

Fig. 5. Schematic figure of respective axial levels dissected for cranial and trunk crestospheres, and the main differences in the culture medium composition.

interpreted as neural crest cell fate. We also used double immunostaining of PAX7 and SOX9 to confirm the neural crest identity in our trunk crestospheres since these markers show overlapping expression only in the neural crest. Based on the expression of neuroepithelial SOX2, a marker that is also expressed in the neural crest at low levels (Kerosuo et al., 2018), we can however not exclude the possibility of the existence of some dorsal neural stem cells, a cell type that in the embryo naturally occurs very close to neural crest cells in the dorsal neural tube (Lignell et al., 2017). Many of the known neural crest factors (*e.g.* SOX10, SOX9, AP2alpha, SNAI2 and PAX7) are expressed from premigratory to late migratory stages, and their expression does not automatically reflect a certain developmental time point. However, other markers, such as FOXD3 (premigratory to very early stage migration) or HNK1 (encoded by B3GAT1) and NGFR (migratory) are more specific and can be used to distinguish between these different stages. Based on our data, we conclude that the trunk crestosphere cultures are a mixture of stem- (premigratory) as well as more differentiated progenitor cell types of trunk axial level neural crest. In addition, based on enriched expression of a set of genes shown by a recent study to be upregulated in trunk neural crest by performing a trunk-specific SOX10-enhancer based RNAseq screen (Murko et al., 2018), the expression profile of our spheres demonstrates a dominance of trunk axial level as compared to cranial crestospheres.

Analysis of trunk crestosphere gene expression at two and four weeks of culture suggests that the premigratory neural crest cell state was maintained for at least one month of culture. Considering that the neural crest is a transient population of stem cells that is maintained in the premigratory stage only for less than a day, we conclude that the conditions used in our trunk cultures maintain neural crest stem cell potential for extended periods of time.

We show that trunk crestospheres are self-renewing neuroepithelial cells enriched for neural crest. Furthermore, differentiation assays show that trunk crestospheres are multipotent with the ability to differentiate into multiple lineages including neurons, glia, smooth muscle (Bittencourt et al., 2013), and to our surprise, also a few cells of the cartilage lineage. Since all the cultures were originated from trunk axial levels that do not form neural crest-derived cartilage in the embryo, this data may reflect a difference between the *in vitro* and *in vivo* potential. Alternatively, the potential can be equal in both, but the trigger for the inhibition of the cartilage potential present in the embryo may be missing from our cultures. Finally, even though we have demonstrated that crestosphere conditions do not support maintenance of a mesodermal fate, low levels of contamination followed by successful maintenance of some somite-derived cells cannot be ruled out, and verification of this interesting finding thus requires further proof.

While providing a useful tool for studying normal neural crest development, trunk crestosphere cultures also provide a novel approach for advancing research on neural crest-derived diseases and malignancies, including neuroblastoma, paraganglioma and pheochromocytoma, as well as syndromes like Congenital Central Hypoventilation Syndrome (CCHS), Familial Dysautonomia (FD), or von Hippel-Lindau Syndrome (Huber et al., 2018; Tsubota and Kadomatsu, 2018; Zhang et al., 2010). Given the ability to perform viral transduction, trunk crestospheres further promise to provide insight into the molecular mechanisms that govern normal development as well as diseases derived from the more posterior parts of the embryo.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2019.01.010.

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